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UNITED STATES PATENT APPLICATION

OF

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FOR

POLYNUCLEOTIDES AND THEIR USE FOR DETECTING RESISTANCE TO
STREPTOGRAMIN A OR TO STREPTOGRAMIN B AND RELATED COMPOUNDS

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application hereby claims the benefit under 35 U.S.C. § 119(e) of United States provisional application S.N. 60/050,380, filed June 20, 1997. The entire disclosure of this application is relied upon and incorporated by reference herein.

BACKGROUND OF THE INVENTION

The present invention pertains to polynucleotides derived from *staphylococcal* genes encoding resistance to streptogramin A or to streptogramin B and chemically related compounds. This invention also relates to the use of the polynucleotides as oligonucleotide primers or probes for detecting *Staphylococcal* strains that are resistant to streptogramin A or to streptogramin B and related compounds in a biological sample.

In another embodiment, the present invention is directed to the full length coding sequences of the *staphylococcal* genes encoding for resistance to streptogramin A or to streptogramin B from *Staphylococcus* and to the polypeptides expressed by these full length coding sequences.

Further, this invention relates to the use of the expressed polypeptides to produce specific monoclonal or polyclonal antibodies that serve as detection means in order to characterize any *staphylococcal* strain carrying genes encoding resistance to streptogramin A or to streptogramin B.

The present invention is also directed to diagnostic methods for detecting specific strains of *Staphylococcus* expected to be contained in a biological sample. The diagnostic methods use the oligonucleotide probes and primers as well as the antibodies of the invention.

Streptogramins and related compounds (antibiotics) produced by streptomycetes can be classified as A and B compounds according to their basic primary structures (Cocito, 1979). Compounds of the A group, including streptogramin A (SgA), pristinamycin IIA (PIIA), virginiamycin M, mikamycin A, or synergistin A, are polyunsaturated cyclic macrolactones. Compounds of the B group, including streptogramin B (SgB), pristinamycin B (PIB), virginiamycin S, mikamycin B, and synergistin B, are cyclic peptidic macrolactones (Cocito, 1979). Compounds of both groups, A and B, bind different targets in the peptidyltransferase domain of the 50S ribosomal subunit and inhibit protein elongation at different steps (Aumercier et al., 1992; Di Giambattista et al., 1989).

A decrease in the dissociation constant of PIB is observed in the presence of PIIA because this latter antibiotic provokes a conformational modification of the bacterial ribosome at the binding sites of these molecules. Thus, A and B compounds, which are bacteriostatic when used separately, act synergistically when

combined and become bactericidal, mainly against Gram-positive bacteria.

Natural mixtures such as pristinamycin (Pt), synergistin, virginiamycin and mikamycin, are used orally and topically. A semi-synthetic injectable streptogramin, RP59500, consisting of a mixture of derivatives of A and B compounds (Dalfopristin and Quinupristin, respectively) is currently undergoing *in vivo* experimental and clinical trials (J. Antimicrob. Agents Chemother. 30 (Suppl. A), entire volume, 1992; Entenza et al., 1995; Fantin et al., 1995; Griswold et al., 1996; Torralba et al., 1995). Staphylococcal resistance to synergistic mixtures of A and B compounds (Pt MIC ≥ 2 $\mu\text{g/ml}$) is always associated with resistance to A compounds (PIIA MIC ≥ 8 $\mu\text{g/ml}$), but not necessarily with resistance to B compounds (Allignet et al., 1996).

To date, four genes encoding resistance to A compounds have been isolated from staphylococcal and enterococcal plasmids. The genes *vat* (Allignet et al., 1993), *vatB* (Allignet and El Solh, 1995), and *satA* (Rende-Fournier et al., 1993) encode related acetyltransferases (50.4-58.3 % amino acids), which inactivate streptogramin A and similar compounds. The staphylococcal gene *vga* (Allignet et al., 1992) encodes an ATP-binding protein probably involved in the active efflux of A compounds.

Nevertheless, there continues to exist a need in the art for

polynucleotides specific for *Staphylococcus* resistant to streptogramin A and/or B and related compounds.

SUMMARY OF THE INVENTION

Accordingly, this invention aids in fulfilling this need in the art. In particular, this invention provides a purified peptide comprising an amino acid sequence selected from the group consisting of :

- a) SEQ ID NO: 4 which corresponds to the complete amino acid sequence of Vga B or fragments derived from SEQ ID NO: 4 containing at least 10 amino acids;
- b) SEQ ID NO: 5 which corresponds to the complete amino acid sequence of Vat C or fragments derived from SEQ ID NO: 5 containing at least 10 amino acids;
- c) SEQ ID NO: 6 which corresponds to the complete amino acid sequence of Vgb B or fragments derived from SEQ ID NO: 6 containing at least 10 amino acids;
- d) SEQ ID NO: 7 which corresponds to a fragment of the amino acid sequence of Vgb B;
- e) SEQ ID NO: 8 which corresponds to a fragment of the amino acid sequence of Vga B;
- f) SEQ ID NO: 9 which corresponds to a fragment of the amino acid sequence of Vat C; and

g) SEQ ID NO: 10 which corresponds to a fragment of the amino acid sequence of Vat C.

This invention additionally provides a purified polynucleotide comprising the nucleotide sequence selected from the group consisting of:

- a) SEQ ID NO: 1 which corresponds to the complete nucleic acid sequence of vga B or fragments derived from SEQ ID NO: 1 containing 15 to 40 nucleotides;
- b) SEQ ID NO: 2 which corresponds to the complete nucleic acid sequence of vat C or fragments derived from SEQ ID NO: 2 containing 15 to 40 nucleotides;
- c) SEQ ID NO: 3 which corresponds to the complete nucleic acid sequence of vgb B or fragments derived from SEQ ID NO: 3 containing 15 to 40 nucleotides;
- d) SEQ ID NO: 11 which corresponds to the nucleic acid sequence encoding the polypeptide of SEQ ID NO: 7;
- e) SEQ ID NO: 12 which corresponds to the nucleic acid sequence encoding the polypeptide of SEQ ID NO: 8 ;
- f) SEQ ID NO: 13 which corresponds to the nucleic acid sequence encoding the polypeptide of SEQ ID NO: 9; and
- g) SEQ ID NO: 14 which corresponds to the nucleic acid sequence encoding the polypeptide of SEQ ID NO: 10.

Furthermore, this invention includes a purified peptide comprising the amino acid sequence encoded by the nucleotide sequence selected from the group consisting of:

- a) SEQ ID NO: 1,
- b) SEQ ID NO: 2,
- c) SEQ ID NO: 3,
- d) SEQ ID NO: 11,
- e) SEQ ID NO: 12,
- f) SEQ ID NO: 13, and
- g) SEQ ID NO: 14.

This invention also provides a composition comprising purified polynucleotide sequences including at least one nucleotide sequence of the genes selected from the group consisting of polypeptides or genes or cDNA of *vgaB*, *vatC*, and *vgbB*, which are useful for the detection of resistance to streptogramin A or to streptogramin B and related compounds.

In another embodiment, this invention provides a composition of polynucleotide sequences encoding resistance to streptogramins and related compounds, or inducing this resistance in Gram-positive bacteria, wherein the composition comprises a combination of at least two of the following nucleotide sequences: a) a nucleotide sequence encoding an acetyltransferase conferring resistance to streptogramin A and related compounds, b) a nucleotide sequence encoding a molecule containing ATP

binding motifs conferring resistance to streptogramin A and related compounds; and c) a nucleotide sequence encoding a lactonase conferring resistance to streptogramin B and related compounds.

Furthermore, this invention provides a composition of polynucleotide sequences, wherein the sequence encoding a molecule containing ATP binding motifs confers resistance to *Staphylococci* and particularly to *S. aureus*, and wherein the polynucleotide sequence corresponds to a *vgaB* nucleotide sequence represented by SEQ ID NO: 1 or a sequence having at least 70% homology with *vgaB* complete nucleotide sequence, or to a polynucleotide hybridizing with SEQ ID NO: 1 under stringent conditions, or to a fragment containing between 20 and 30 nucleotides of SEQ ID NO: 11 or SEQ ID NO: 12, or wherein the polynucleotide sequence encodes a polypeptide having at least 60% homology with the complete SEQ ID NO: 4 or with SEQ ID NO: 7 or SEQ ID NO: 8.

Furthermore this invention relates to a composition of polynucleotide sequences, wherein the sequence encoding an acetyltransferase confers resistance to streptogramin A and related compounds in *Staphylococci*, and particularly in *S. cohnii*, and wherein the polynucleotide sequence corresponds to a *vatC* nucleotide sequence represented by SEQ ID NO: 2 or a sequence having at least 70% homology with *vatC* complete

nucleotide sequence, or to a polynucleotide hybridizing with SEQ ID NO: 2 under stringent conditions, or to a fragment containing between 20 and 30 nucleotides of SEQ ID NO: 13 or SEQ ID NO: 14, or wherein the polynucleotide sequence encodes a polypeptide having at least 60% homology with the complete SEQ ID NO: 5 or with SEQ ID NO: 9 or SEQ ID NO: 10.

This invention also provides a composition of polynucleotide sequences, wherein the sequence encoding a lactonase confers resistance to streptogramin B and related compounds in *Staphylococci* and particularly in *S. cohnii*, and wherein the polynucleotide sequence corresponds to a *vgbB* nucleotide sequence represented in SEQ ID NO: 3 or a sequence having at least 70% homology with *vgbB* complete nucleotide sequence, or to a polynucleotide hybridizing with SEQ ID NO: 3 under stringent conditions, or to a fragment containing between 20 and 40 nucleotides of SEQ ID NO: 3, or wherein the polynucleotide sequence encodes a polypeptide having at least 60% homology with the complete SEQ ID NO: 6.

The invention also contemplates a composition of polynucleotide sequences, wherein at least a *vatB* nucleotide sequence encoding an acetyltransferase conferring resistance to streptogramin A and related compounds is included in addition to a *vgaB* nucleotide sequence encoding a molecule containing ATP binding motifs conferring resistance to streptogramin A.

Additionally, the invention includes a purified polynucleotide that hybridizes specifically under stringent conditions with a polynucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, and SEQ ID NO: 14.

The invention further includes polynucleotide fragments comprising at least 10 nucleotides capable of hybridization under stringent conditions with any one of the nucleotide sequences enumerated above.

In another embodiment of the invention, a recombinant DNA sequence comprising at least one nucleotide sequence enumerated above and under the control of regulatory elements that regulate the expression of resistance to antibiotics of the streptogramin family in a defined host is provided.

Furthermore, the invention includes a recombinant vector comprising the recombinant DNA sequence noted above, wherein the vector comprises the plasmid pIP1633 or plasmid pIP1714.

The invention also includes a recombinant cell host comprising a polynucleotide sequence enumerated above or the recombinant vector defined above.

In still a further embodiment of the invention, a method of detecting bacterial strains that contain the polynucleotide sequences set forth above is provided.

Additionally, the invention includes kits for the detection of the presence of bacterial strains that contain the polynucleotide sequences set forth above.

The invention also contemplates antibodies recognizing peptide fragments or polypeptides encoded by the polynucleotide sequences enumerated above.

Still further, the invention provides for a screening method for active antibiotics and/or molecules for the treatment of infections due to Gram-positive bacteria, particularly staphylococci, based on the detection of activity of these antibiotics and/or molecules on bacteria having the resistance phenotype to streptogramins.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

This invention will be more fully described with reference to the drawings in which:

FIG. 1A and 1B are the restriction maps of the 5.5 kb *Bgl*II fragment and of the 2.4 kb *Hind*III-*Hae*III fragment of pIP1633, respectively. Both fragments confer resistance to streptogramin A and related compounds. The strategy for sequencing the 2.4 kb *Hind*III-*Hae*III fragment is given in Fig. 1B. Restriction enzyme

abbreviations: Ba, *Bam*HI; Bg, *Bg*III; E, *Eco*RI; H, *Hind*III; X, *Xba*I.

FIG. 2 is the nucleotide sequence and deduced amino acid sequence of 2411 nucleotides from pIP1633, which contains the gene *vgaB* of *S. aureus* conferring resistance to streptogramin A and related compounds. The putative ribosome binding site (RBS) is underlined. The amino acids are aligned with the second nucleotide of each codon. Asterisks indicate the in-frame stop codons. The A and B ATP-binding motifs described by Walker et al. (1982) and detected within each of the two ATP-domains are boxed. The conserved motif SGG of the two copies of loop 3 described by Hyde et al. (1990) is underlined. Relevant restriction sites are shown.

FIG. 3 is the amino acid sequence alignment of the predicted 60 and 61 kDa proteins encoded by *Vga* (Allignet et al., 1992, accession No: m90056) and *VgaB* (FIG.2), respectively. Identical residues are indicated by asterisks and conservative changes are shown by single dots. The A and B motifs of Walker et al. (1982) are in bold type (WA, WB). The conserved motif SGG of the two copies of loop 3 described by Hyde et al. (1990) is underlined.

FIG. 4 is a restriction map of the plasmid pIP1714 carrying the genes *vatC* and *vgbB* as well as the genes *pre* and *repB* of *S.*

cohnii strain BM10711 resistant to the synergistic mixtures of streptogramins A and B.

FIG. 5 is the nucleotide sequence and deduced amino acid sequence of 1727 nucleotide from pIP1714, which contains the gene *vgbB* and *vatC* of *S. cohnii*. Relevant restriction sites are shown.

FIG. 6 A, 6B, and 6C represent oligonucleotide primers for hybridization under stringent conditions with *vatC*, *vgbB*, and *vgaB* respectively.

FIG. 7 represents SEQ ID NOs: 1-14.

DETAILED DESCRIPTION OF THE INVENTION

It has now been determined that bacteria from the *Staphylococcus* genus carry a *vgaB* gene, which encodes a putative ATP-binding protein that confers resistance to streptogramin A and structurally similar compounds. It has also now been determined that bacteria from the *Staphylococcus* genus carry a *vgbB* gene, which encodes a lactonase that confers resistance to streptogramin B and structurally similar compounds, and a *vatC* gene, which encodes an acetyltransferase that confers resistance to streptogramin A and structurally similar compounds.

Novel polynucleotides corresponding to the *vgaB*, *vgbB*, and *vatC* genes from various strains of *Staphylococcus* have been isolated and sequenced, and it has been surprisingly demonstrated

that these new polynucleotides make it possible to design oligonucleotide probes or primers. These polynucleotides include the following:

- a) SEQ ID NO: 1,
- b) SEQ ID NO: 2,
- c) SEQ ID NO: 3,
- d) SEQ ID NO: 11,
- e) SEQ ID NO: 12,
- f) SEQ ID NO: 13, and
- g) SEQ ID NO: 14.

This invention provides specific pairs of oligonucleotide primers or probes that hybridize specifically, under stringent hybridization conditions as defined hereinafter, to the nucleic acid (RNA or DNA) from a particular strain of the *Staphylococcus* genus. These oligonucleotide primers include the following:

- a) Oligo I 5'-AAGTCGACTGACAATATGAGTGGTGG-3'
Oligo II 5'-CTGCAGATGCCTCAACAGCATCGATATCC-3'
- b) Oligo III 5'- ATGAATTCGCAAATCAGCAAGG -3'
Oligo IV 5'- TCGTCTCGAGCTCTAGGTCC -3'
- c) Oligo V 5'- CAGCAGTCTAGATCAGAGTGG -3'
Oligo VI 5'- CATACGGATCCACCTTTTCC -3'.

In a specific embodiment of the present invention, the purified polynucleotides useful for detecting *Staphylococcal* strains can be used in combination in order to detect bacteria

belonging to *Staphylococci* in a biological sample. Thus, the present invention also provides detection methods and kits comprising combinations of the purified polynucleotides according to the invention. The purified oligonucleotides of the invention are also useful as primers for use in amplification reactions or as nucleic acid probes.

By "polynucleotides" according to the invention is meant the sequences referred to as SEQ ID NOs: 1, 2, 3, OR 11, 12, 13, 14 and the complementary sequences and/or the sequences of polynucleotides which hybridize to the referred sequences in high stringent conditions and which are used for detecting *staphylococcal* strains carrying a gene encoding resistance to streptogramin A or to streptogramin B.

By "active molecule" according to the invention is meant a molecule capable of inhibiting the activity of the purified polypeptide as defined in the present invention or capable of inhibiting the bacterial culture of *staphylococcal* strains.

Thus, the polynucleotides of SEQ ID NOs: 1-3 and 11-14 and their fragments can be used to select nucleotide primers notably for an amplification reaction, such as the amplification reactions further described.

PCR is described in the U.S. Patent No. 4,683,202 granted to Cetus Corp. The amplified fragments may be identified by agarose or polyacrylamide gel electrophoresis, or by a capillary

electrophoresis, or alternatively by a chromatography technique (gel filtration, hydrophobic chromatography, or ion exchange chromatography). The specificity of the amplification can be ensured by a molecular hybridization using as nucleic probes the polynucleotides of SEQ ID NOs: 1-3 and 11-14 and their fragments, oligonucleotides that are complementary to these polynucleotides or fragments thereof, or their amplification products themselves.

Amplified nucleotide fragments are useful as probes in hybridization reactions in order to detect the presence of one polynucleotide according to the present invention or in order to detect the presence of a bacteria of *Staphylococcal* strain carrying genes encoding resistance to streptogramin A or streptogramin B, in a biological sample. This invention also provides the amplified nucleic acid fragments ("amplicons") defined herein above. These probes and amplicons can be radioactively or non-radioactively labeled, using for example enzymes or fluorescent compounds.

Preferred nucleic acid fragments that can serve as primers according to the present invention are the following:

polynucleotides of sequence SEQ ID NOs: 1-3 and 11-14;
and

polynucleotides having a length from 20 to 30
consecutive nucleotides from a polynucleotide selected
from the group consisting of polynucleotides of

sequences SEQ ID NO: 11 to SEQ ID NO: 14 or from 20 to 40 consecutive nucleotides from a polynucleotide of SEQ ID NO: 3

The primers can also be used as oligonucleotide probes to specifically detect a polynucleotide according to the invention.

Other techniques related to nucleic acid amplification can also be used and are generally preferred to the PCR technique. The Strand Displacement Amplification (SDA) technique (Walker et al., 1992) is an isothermal amplification technique based on the ability of a restriction enzyme to cleave one of the strands at a recognition site (which is under a hemiphosphorothioate form), and on the property of a DNA polymerase to initiate the synthesis of a new strand from the 3' OH end generated by the restriction enzyme and on the property of this DNA polymerase to displace the previously synthesized strand being localized downstream.

The SDA amplification technique is more easily performed than PCR (a single thermostated water bath device is necessary), and is faster than the other amplification methods. Thus, the present invention also comprises using the nucleic acid fragments according to the invention (primers) in a method of DNA or RNA amplification according to the SDA technique. The polynucleotides of SEQ ID NOs: 1-3 and 11-14 and their fragments, especially the primers according to the invention, are useful as

technical means for performing different target nucleic acid amplification methods such as:

- TAS (Transcription-based Amplification System), described by Kwoh et al. in 1989;
- SR (Self-Sustained Sequence Replication), described by Guatelli et al. in 1990;
- NASBA (Nucleic acid Sequence Based Amplification), described by Kievitis et al. in 1991; and
- TMA (Transcription Mediated Amplification).

The polynucleotides of SEQ ID NOs: 1-3 and 11-14 and their fragments, especially the primers according to the invention, are also useful as technical means for performing methods for amplification or modification of a nucleic acid used as a probe, such as:

- LCR (Ligase Chain Reaction), described by Landegren et al. in 1988 and improved by Barany et al. in 1991, who employ a thermostable ligase;
- RCR (Repair Chain Reaction), described by Segev et al. in 1992;
- CPR (Cycling Probe Reaction), described by Duck et al. in 1990; and
- Q-beta replicase reaction, described by Miele et al. in 1983 and improved by Chu et al. in 1986, Lizardi et al. in 1988, and by Burg et al. and Stone et al. in 1996.

When the target polynucleotide to be detected is RNA, for example mRNA, a reverse transcriptase enzyme can be used before the amplification reaction in order to obtain a cDNA from the RNA contained in the biological sample. The generated cDNA can be subsequently used as the nucleic acid target for the primers or the probes used in an amplification process or a detection process according to the present invention.

Nucleic probes according to the present invention are specific to detect a polynucleotide of the invention. By "specific probes" according to the invention is meant any oligonucleotide that hybridizes with one polynucleotide of SEQ ID NOs: 1-3 and 11-14 and which does not hybridize with unrelated sequences. Preferred oligonucleotide probes according to the invention are oligonucleotides I-VI.

In a specific embodiment, the purified polynucleotides according to the present invention encompass polynucleotides having at least 80% homology in their nucleic acid sequences with polynucleotides of SEQ ID NO: 11 to SEQ ID NO: 14, at least 70% identity with SEQ ID NO: 1 to 3. By percentage of nucleotide homology according to the present invention is intended a percentage of identity between the corresponding bases of two homologous polynucleotides, this percentage of identity being purely statistical and the differences between two homologous

polynucleotides being located at random and on the whole length of said polynucleotides.

The oligonucleotide probes according to the present invention hybridize specifically with a DNA or RNA molecule comprising all or part of one polynucleotide among SEQ ID NOs: 1-3 and 11-14 under stringent conditions. As an illustrative embodiment, the stringent hybridization conditions used in order to specifically detect a polynucleotide according to the present invention are advantageously the following:

Prehybridization and hybridization are performed at 68°C in a mixture containing:

- 5X SSPE (1X SSPE is .3 M NaCl, 30 mM tri-sodium citrate
- 5X Denhardt's solution
- 0.5% (w/v) sodium dodecyl sulfate (SDS); and
- 100 µg ml⁻¹ salmon sperm DNA

The washings are performed as follows:

- Two washings at laboratory temperature for 10 min. in the presence of 2 x SSPE and 0.1 % SDS;
- One washing at 68°C for 15 min. in the presence of 1 x SSPE, .1% SDS; and
- One washing at 68°C for 15 min. in the presence of 0.1 x SSPE and 0.1 % SDS.

The non-labeled polynucleotides or oligonucleotides of the invention can be directly used as probes. Nevertheless, the polynucleotides or oligonucleotides are generally labeled with a radioactive element (^{32}P , ^{35}S , ^3H , ^{125}I) or by a non-isotopic molecule (for example, biotin, acetylaminofluorene, digoxigenin, 5-bromodesoxyuridin, fluorescein) in order to generate probes that are useful for numerous applications. Examples of non-radioactive labeling of nucleic acid fragments are described in the French Patent No. FR 78 10975 or by Urdea et al. or Sanchez-Pescador et al. 1988.

Other labeling techniques can also be used, such as those described in the French patents 2 422 956 and 2 518 755. The hybridization step may be performed in different ways (Matthews et al. 1988). A general method comprises immobilizing the nucleic acid that has been extracted from the biological sample on a substrate (nitrocellulose, nylon, polystyrene) and then incubating, in defined conditions, the target nucleic acid with the probe. Subsequent to the hybridization step, the excess amount of the specific probe is discarded, and the hybrid molecules formed are detected by an appropriate method (radioactivity, fluorescence, or enzyme activity measurement).

Advantageously, the probes according to the present invention can have structural characteristics such that they allow signal amplification, such structural characteristics

being, for example, branched DNA probes as those described by Urdea et al. in 1991 or in the European Patent No. 0 225 807 (Chiron).

In another advantageous embodiment of the present invention, the probes described herein can be used as "capture probes", and are for this purpose immobilized on a substrate in order to capture the target nucleic acid contained in a biological sample. The captured target nucleic acid is subsequently detected with a second probe, which recognizes a sequence of the target nucleic acid that is different from the sequence recognized by the capture probe.

The oligonucleotide fragments useful as probes or primers according to the present invention can be prepared by cleavage of the polynucleotides of SEQ ID NOs: 1-3 and 11-14 by restriction enzymes, as described in Sambrook et al. in 1989. Another appropriate preparation process of the nucleic acids of the invention containing at most 200 nucleotides (or 200 bp if these molecules are double-stranded) comprises the following steps:

- synthesizing DNA using the automated method of beta-cyanethylphosphoramidite described in 1986;
- cloning the thus obtained nucleic acids in an appropriate vector; and
- purifying the nucleic acid by hybridizing to an appropriate probe according to the present invention.

A chemical method for producing the nucleic acids according to the invention, which have a length of more than 200 nucleotides (or 200 bp if these molecules are double-stranded), comprises the following steps:

- Assembling the chemically synthesized oligonucleotides having different restriction sites at each end;
- cloning the thus obtained nucleic acids in an appropriate vector; and
- purifying the nucleic acid by hybridizing to an appropriate probe according to the present invention.

The oligonucleotide probes according to the present invention can also be used in a detection device comprising a matrix library of probes immobilized on a substrate, the sequence of each probe of a given length being localized in a shift of one or several bases, one from the other, each probe of the matrix library thus being complementary to a distinct sequence of the target nucleic acid. Optionally, the substrate of the matrix can be a material able to act as an electron donor, the detection of the matrix positions in which hybridization has occurred being subsequently determined by an electronic device. Such matrix libraries of probes and methods of specific detection of a target nucleic acid are described in the European patent application No. 0 713 016, or PCT Application No. WO 95 33846, or also PCT

Application No. WO 95 11995 (Affymax Technologies), PCT Application No. WO 97 02357 (Affymetrix Inc.), and also in U.S. Patent No. 5,202,231 (Drmanac), said patents and patent applications being herein incorporated by reference.

The present invention also pertains to a family of recombinant plasmids containing at least a nucleic acid according to the invention. According to an advantageous embodiment, a recombinant plasmid comprises a polynucleotide of SEQ ID NOs: 1-3 and 11-14 or one nucleic fragment thereof. More specifically, the following plasmids are part of the invention: pIP1633 and pIP1714.

The present invention is also directed to the full length coding sequences of the *vgaB*, *vgbB*, and *vatC* genes from *Staphylococci* that are available using the purified polynucleotides according to the present invention, as well as to the polypeptide enzymes encoded by these full length coding sequences. In a specific embodiment of the present invention, the full length coding sequences of the *vgaB*, *vgbB*, and *vatC* genes are isolated from a plasmid or cosmid library of the genome of *Staphylococci* that have been screened with the oligonucleotide probes according to the present invention. The selected positive plasmid or cosmid clones hybridizing with the oligonucleotide probes of the invention are then sequenced in order to characterize the corresponding full length coding sequence, and

the DNA insert of interest is then cloned in an expression vector in order to produce the corresponding ATP binding motif conferring resistance to streptogramin A and related compounds, acetyltransferase conferring resistance to streptogramin A and related compounds, or lactonase conferring resistance to streptogramin B and related compounds.

A suitable vector for the expression in bacteria and in particular in *E. coli*, is the pQE-30 vector (QIAexpress) that allows the production of a recombinant protein containing a 6xHis affinity tag. The 6xHis tag is placed at the C-terminus of the recombinant polypeptide ATP binding motif conferring resistance to streptogramin A and related compounds, acetyltransferase conferring resistance to streptogramin A and related compounds or lactonase conferring resistance to streptogramin B and related compounds, which allows a subsequent efficient purification of the recombinant polypeptide ATP binding motif conferring resistance to streptogramin A and related compounds, acetyltransferase conferring resistance to streptogramin A and related compounds, or lactonase conferring resistance to streptogramin B and related compounds by passage onto a nickel or copper affinity chromatography column. The nickel chromatography column can contain the Ni-NTA resin (Porath et al. 1975).

The polypeptides according to the invention can also be prepared by conventional methods of chemical synthesis, either in

a homogenous solution or in solid phase. As an illustrative embodiment of such chemical polypeptide synthesis techniques the homogenous solution technique described by Houbenweyl in 1974 may be cited.

The polypeptides according to the invention can be characterized by binding onto an immunoaffinity chromatography column on which polyclonal or monoclonal antibodies directed to a polypeptide among the ATP binding motif conferring resistance to streptogramin A and related compounds, acetyltransferase conferring resistance to streptogramin A and related compounds, or lactonase conferring resistance to streptogramin B and related compounds of the invention have previously been immobilized.

Another object of the present invention comprises a polypeptide produced by the genetic engineering techniques or a polypeptide synthesized chemically as above described.

The polypeptide ATP binding motif conferring resistance to streptogramin A and related compounds, acetyltransferase conferring resistance to streptogramin A and related compounds, or lactonase conferring resistance to streptogramin B and related compounds according to the present invention are useful for the preparation of polyclonal or monoclonal antibodies that recognize the polypeptides or fragments thereof. The monoclonal antibodies can be prepared from hybridomas according to the technique described by Kohler and Milstein in 1975. The polyclonal

antibodies can be prepared by immunization of a mammal, especially a mouse or a rabbit, with a polypeptide according to the invention that is combined with an adjuvant, and then by purifying specific antibodies contained in the serum of the immunized animal on a affinity chromatography column on which has previously been immobilized the polypeptide that has been used as the antigen.

Consequently, the invention is also directed to a method for detecting specifically the presence of a polypeptide according to the invention in a biological sample. The method comprises:

- a) bringing into contact the biological sample with an antibody according to the invention; and
- b) detecting antigen-antibody complex formed.

Also part of the invention is a diagnostic kit for *in vitro* detecting the presence of a polypeptide according to the present invention in a biological sample. The kit comprises:

- a polyclonal or monoclonal antibody as described above, optionally labeled; and
- a reagent allowing the detection of the antigen-antibody complexes formed, wherein the reagent carries optionally a label, or being able to be recognized itself by a labeled reagent, more particularly in the case when the above-mentioned

monoclonal or polyclonal antibody is not labeled by itself.

Indeed, the monoclonal or polyclonal antibodies according to the present invention are useful as detection means in order to identify or characterize a *Staphylococcal* strain carrying genes encoding resistance to streptogramin A or streptogramin B.

The invention also pertains to:

A purified polypeptide or a peptide fragment having at least 10 amino acids, which is recognized by antibodies directed against a polynucleotide sequence conferring resistance to streptogramin and related compounds, corresponding to a polynucleotide sequence according to the invention.

A polynucleotide comprising the full length coding sequence of a *Staphylococcus* streptogramin A and/or B resistant gene containing a polynucleotide sequence according to the invention.

A monoclonal or polyclonal antibody directed against a polypeptide or a peptide fragment encoded by the polynucleotide sequences according to the invention.

A method of detecting the presence of bacterium harboring the polynucleotide sequences according to the invention in a biological sample comprising:

- a) contacting bacterial DNA of the biological sample with a primer or a probe according to the

- invention, which hybridizes with a nucleotide sequence encoding resistance to streptogramins;
- b) amplifying the nucleotide sequence using said primer or said probe; and
 - c) detecting the hybridized complex formed between said primer or probe with the DNA.

A kit for detecting the presence of bacterium having resistance to streptogramin A and/or streptogramin B and harboring the polynucleotide sequences according to the invention in a biological sample, said kit comprising:

- a) a polynucleotide probe according to the invention; and
- b) reagents necessary to perform a nucleic acid hybridization reaction.

A kit for detecting the presence of bacterium having resistance to streptogramin A and harboring the polynucleotide sequences according to the invention in a biological sample, said kit comprising:

- a) a polynucleotide probe according to the invention; and
- b) reagents necessary to perform a nucleic acid hybridization reaction.

A method of screening active antibiotics for the treatment of the infections due to Gram-positive bacteria, comprising the steps of:

- a) bringing into contact a Gram-positive bacteria having a resistance to streptogramin A or streptogramin B and related compounds and containing the polynucleotide sequences according to the invention with the antibiotic; and
- b) measuring an activity of the antibiotic on the bacteria having a resistance to streptogramins and related compounds.

A method of screening for active synthetic molecules capable of penetrating into a bacteria of the family of staphylococci, wherein the inhibiting activity of these molecules is tested on at least a polypeptide encoded by the polynucleotide sequences according to the invention comprising the steps of:

- a) contacting a sample of said active molecules with the bacteria;
- b) testing the capacity of the active molecules to penetrate into the bacteria and the capacity of inhibiting a bacterial culture at various concentration of the molecules; and

- c) choosing the active molecule that provides an inhibitory effect of at least 80% on the bacterial culture compared to an untreated culture.

An *in vitro* method of screening for active molecules capable of inhibiting a polypeptide encoded by the polynucleotide sequences according to the invention, wherein the inhibiting activity of these molecules is tested on at least said polypeptide, said method comprising the steps of:

- a) extracting a purified polypeptide according to the invention;
- b) contacting the active molecules with said purified polypeptide;
- c) testing the capacity of the active molecules, at various concentrations, to inhibit the activity of the purified polypeptide; and
- d) choosing the active molecule that provides an inhibitory effect of at least 80 % on the activity of the said purified polypeptide.

A composition of a polynucleotide sequence encoding resistance to streptogramins and related compounds, or inducing resistance in Gram-positive bacteria, wherein said composition comprises a nucleotide sequence corresponding to the resistance phenotype of the plasmid pIP1633 deposited with the C.N.C.M. under the Accession No. I-1768 and of the plasmid pIP1680

deposited with the C.N.C.M. under the Accession No. I-1767 and of the plasmid pIP1714 deposited with the C.N.C.M. under the number I-1877 on June 18, 1997.

A method of detecting the presence of bacterium harboring the polynucleotide sequences according to the invention in a biological sample, said method comprising the steps of:

- a) contacting said sample with an antibody according to the invention that recognizes a polypeptide encoded by said polynucleotide sequences; and
- b) detecting said complex.

A diagnostic kit for *in vitro* detecting the presence of bacterium harboring the polynucleotide sequences according to the invention in a biological sample, said kit comprising:

- a) a predetermined quantity of monoclonal or polyclonal antibodies according to the invention;
- b) reagents necessary to perform an immunological reaction between the antibodies and a polypeptide encoded by said polynucleotide sequences; and
- c) reagents necessary for detecting said complex between the antibodies and the polypeptide encoded by said polynucleotide sequences.

The inhibiting activity of the molecules can be readily evaluated by one skilled in the art. For example, the inhibiting

activity of Vga B can be tested by detecting its ATP hydrolysis as described in J.I. Ross et al. (1990), Mol. Microbiol. 4(7):1207-1214 regarding the rate evaluation of the active efflux of antibiotics from a cell. Ross et al. use a different gene, but their gene product functions as a drug efflux pump in the same way as Vga B does.

The inhibiting activity of Vat C can be tested by visualizing the acetylation reaction as described in Allignet et al. (1993) regarding the mechanism of inactivation of A-type compounds conferred by plasmids pIP680 and pIP1156 by thick layer chromatography and NMR.

The inhibiting activity of Vgb B can be tested by detecting the degradation of streptogramin B or a related compound by a microbiological test as described in Allignet et al. (1988).

Plasmids containing the polynucleotides from *Staphylococci*, which confer streptogramin A and/or B resistance, are referred to herein by the following accession numbers:

<u>Plasmid</u>	<u>Accession No.</u>
pIP1714	I- 1877
pIP1633	I-1768
pIP680	I-1767

and they have been inserted into vectors which have been deposited at the Collection Nationale de Cultures de Microorganismes ("C.N.C.M.") Institut Pasteur, 28, rue du Docteur

Roux, F-75724 Paris Cedex 15, France on June 18, 1997, and August 7, 1996, respectively.

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EXAMPLES

Example 1: Cloning of the *vgaB* gene carried by plasmid pIP1633

pIP1633 was isolated from a *S. aureus* transconjugant strain, BM12235, obtained from the donor wild-type *S. aureus* strain, BM3385 (Allignet and El Solh, 1995). This plasmid carried the *vatB* gene located on a 5.5 *Bgl*III fragment, but the other described streptogramin A resistant (*SgA^r*) genes were not detected either by hybridization experiments or by PCR (Allignet and El Solh, 1995). Since the gene *vga* was carried by all the tested staphylococcal plasmids containing the *vat* gene (Allignet et al., 1996), the presence of a *vga*-related gene was suspected in pIP1633. We therefore searched this gene in the recombinant plasmid, pIP1675 (Fig. 1A), containing the *vatB*-5.5 *Bgl*III fragment of pIP1633.

First, the 2.4 kb *Hind*III-*Hae*III fragment of pIP1675, which contains only 10 nucleotide from *vatB*, was inserted into plasmid pOX300, and the recombinant plasmid, pIP1717 (Fig. 1B), was introduced by electroporation into the *S. aureus* recipient, RN4220 (Kreiwirth et al., 1983). Plasmid pOX300, also named pOX7, (Dyke and Curnock, 1989), is a hybrid of pUC18 and pE194ts and replicates in *E. coli* where it confers resistance to ampicillin and to erythromycin, and in *S. aureus* where only resistance to erythromycin is expressed. The *S. aureus*

transformants selected on 10 μ g/ml erythromycin were resistant to streptogramin A and related compounds (PIIA MICs = 8-16 μ g/ml). Thus, the 2.4-kb *Hind*III-*Hae*III insert of pIP1717 (Fig. 1B) probably carried a streptogramin A resistance gene and was sequenced. The nucleotide (nucleotide) sequence of this fragment was determined by the dideoxy method (Sanger et al., 1977) with the reagents and the procedure recommended by the suppliers of the T⁷ sequencing kit (Pharmacia International). Arrows indicate the direction and extent of each dideoxy-sequencing reaction. (Fig. 1B).

Example 2: The nucleotide sequence of the *vgaB* gene

The strategy of sequencing on both strands is outlined in Fig. 1 and the sequence of the 2411-bp *Hind*III-*Hae*III insert is given in Fig. 2. An open reading frame (ORF) of 1674 nucleotide extending from nucleotide 682 to 2356 was detected on the same strand as *vatB* (Fig. 2). The 1674 nucleotide ORF contained an ATG start codon at nucleotide 700 to 702 and was preceded by an 8 nucleotide putative RBS. The ΔG (free energy of association) of interaction of the most stable structure between this putative RBS and the 3'-terminus of the 16S rRNA (MacLaughlin et al., 1981; Moran et al., 1982) calculated according to Tinoco et al. (1973) was -79.4 kJ/mol. The sequence located between the ATG codon and the TAA stop codon at nucleotide 2356 to 2358 may encode a 552 amino acid protein of 61,327 daltons (Da). This

putative gene, named *vgaB*, had 58.8 % nucleotide identity with the 1572 bp gene, *vga* (Allignet et al., 1992). The G+C content of *vgaB* (27.2 %) is similar to that of *vga* (29 %), but both values are slightly lower than those of the staphylococcal genome (32 to 36 %) (Kloos and Schleifer, 1986). The nucleotide sequence of *vgaB* has been submitted to the GenBank/EMBL data bank under accession no. u82085.

Example 3: *Amino acid sequence analysis of VgaB*

The predicted translation product of the *vgaB* gene, VgaB, has a calculated isoelectric point (pI) of 9.60. The hydropathy plot of the VgaB sequence according to the algorithm of Kyte and Doolittle (1982) indicates the protein to be hydrophilic. No similarity to known signal sequences of secreted proteins (von Heijne, 1986; Watson, 1984) was observed.

The amino acid sequence of VgaB was compared with the sequences available in databases (GenBank, release 97.0; EMBL, release 48; SwissProt, release 34). Significant similarity to the ATP-binding domains of numerous ATP-binding Cassette (ABC) proteins was found. The protein giving the best match was Vga (48.3 % identical amino acid, 70.4 % similar amino acid). VgaB and Vga each contain two ATP-binding domains sharing 38.8 % and 39.1 % identical amino acid, respectively. Each of these domains includes the two ATP-binding motifs described by Walker et al. (1982) (Fig.2). Moreover, the highly conserved SGG sequence of

loop 3 found between the two ATP-binding motifs of all investigated ATP-binding proteins (Barrasa et al., 1995; Hyde et al., 1990) was detected in Vga (Allignet et al., 1992) and VgaB (Fig. 2). According to the predicted tertiary structure of ABC model cassette, this loop would be conveniently located to interact with the cell membrane (Hyde et al., 1990). The inter-ATP-binding domain of VgaB is more rich in glutamine (11 Q in 155 amino acid total) than the rest of the sequence of the protein (11 Q/397 amino acid). In contrast, the proportion of glutamine in the inter-ATP-binding domain of Vga is similar to that in the other part of the protein (4 Q/156 amino acid and 14 Q/366 amino acid, respectively). Neither Vga nor VgaB contains hydrophobic transmembrane domains.

The ABC protein MsrA (Ross et al., 1990) is the most similar to Vga and VgaB (35.2 % and 34.4 % identical amino acid, respectively). MsrA confers resistance to erythromycin by increasing the efflux of this antibiotic and to streptogramin B by a mechanism not yet elucidated. MsrA contains two ATP-binding domains with 31.8% amino acid identity and separated by a Q-linker, but no hydrophobic stretches that might be potential membrane spanning domains. The hydrophobic proteins, which are expected to interact with MsrA, are those encoded by similar genes mapping near MsrA in two staphylococcal strains (*smpA*, *smpB*) and also those on the chromosome of the *S. aureus* recipient

strain, RN4220 (*smpC*), which does not carry *msrA* (Ross et al., 1995). Ross et al. (1996) have recently reported that *SmpC* found in the chromosome of RN4220 is not essential for the expression of resistance to erythromycin conferred by *MsrA*. Thus, further experiments are required to elucidate the mechanisms of resistance conferred by *msrA*, *vga*, or *vgaB* genes.

Several ABC transporters, which do not have alternating hydrophobic domains, have been grouped in a subfamily in order to distinguish them from the members of the ABC₂ transporter subfamily, the members of which contain hydrophobic transmembrane domains (Barrasa et al., 1995; Olano et al., 1995; Peschke et al., 1995). Thus, *VgaB* may be considered as a new member of the former ABC transporter subfamily. Excluding *VgaB*, *Vga*, and *MsrA*, most of the known ABC transporters that contain two ATP-binding cassettes but no hydrophobic domain(s) were found in antibiotic or antibiotic producing microorganisms in which they are involved in the active excretion of these molecules. These transporters are encoded by the following genes: *ard1*, an amino-acylnucleoside antibiotic resistance gene from *Streptomyces capreolus* (Barrasa et al., 1995); *carA*, a carbomycin-resistance gene from *Streptomyces thermotolerans* (Schoner et al., 1992); *ImrC*, a lincomycin-resistance gene from *Streptomyces lincolnensis* (Peschke et al., 1995); *oleB*, an oleandomycin-resistance gene from *Streptomyces antibioticus* (Olano et al., 1995); *srmB*, a

spiramycin-resistance gene from *Streptomyces ambofaciens* (Geistlich et al., 1992); *tlrC*, a tylosin-resistance gene from *Streptomyces fradiae* (Rosteck et al., 1991); and *petT*, a *pep5* epidermin-resistance gene from *Staphylococcus epidermidis* (Meyer et al., 1995). The amino acid identity between each of these latter ABC transporters and *VgaB* is between 23.6 % and 28.7 %.

Degenerate primers designed from an analysis of the alignment of the amino acid sequence of *Vga* and *VgaB* may be helpful to detect such putative genes by PCR experiments. In the streptogramins producers, the described resistance to these antibiotics consists of streptogramin A inactivation by an as yet unknown mechanism (Fierro et al., 1989), streptogramin B inactivation by a lactonase (Kim et al., 1974) and putative increased export of streptogramin A and streptogramin B by an integral membrane protein, *Ptr*, exploiting transmembrane proton gradients (Blanc et al., 1995). The NMR spectra of the modified A compounds may be analyzed to verify if their inactivation in the antibiotic producers is similar to that due to the proteins *Vat* or *VatB*, which transfer an o-acetyl group to position C14 of PIIA (Allignet et al., 1993). Interestingly, the staphylococcal gene *vgb* (Allignet et al., 1988) found in most plasmids carrying *vga* and *vat* (Allignet et al., 1996), encodes a protein inactivating streptogramin B and related compounds by cleavage of the lactone ring.

only in the 21 large plasmids containing *vatB*. In all these 21 plasmids including pIP1633, the hybridizing nucleotide sequences were detected on a 1.5 kb *EcoRI* fragment, which also hybridized with *vatB*, suggesting that *vgaB* and *vatB* have conserved relative positions.

Example 5: Results concerning *vatC* and *vgbB* genes

The *Staphylococcus cohnii* strain, BM10711, resistant to the synergistic mixtures streptogramin A and streptogramin B and related compounds (pristinamycin, virginiamycin, synergistin, mikamycin, Quinupristin-Dalfopristin) was analyzed. This strain was isolated at Douera hospital (Algeria) where the pristinamycin was frequently used topically. The strain was isolated (Liassin et al., 1997) from a sample provided from a cupboard located in a room occupied by patients suffering from chronic osteomyelitis.

The strain BM10711 harbored several plasmids including pIP1714 (5kb). This plasmid was isolated by electroporation in a *S. aureus* recipient strain, RN4220. The transformant, harboring pIP1714, was selected on BHIA containing 10 µg/ml pristinamycin IIA. Plasmid pIP1714 conferred resistances to streptogramin A and streptogramin B and related compounds.

Plasmid pIP1714 was linearized by cleavage with *HindIII* and cloned in the *HindIII* site of the vector pOX7 also named pOX300 (Dyke et al., 1989, FEMS Microbiol. Lett. 58:209-216). pOX7 results from the cointegration of the *E. coli* vector, pUC18, and

S. aureus plasmid, pE194. The recombinant plasmid pIP1715 consisting of pOX7 and pIP1714 was used to sequence pIP1714 in its entirety. The gene *vatC* (636 nucleotides) encoding an acetyltransferase inactivating streptogramin A and related compounds and the gene *vgbB* (885 nucleotides) encoding a lactonase inactivating streptogramin B and related compounds were found to be carried by this plasmid. The gene *vatC* had 71.7, 62.2 and 64.1 % nucleotides identity with *vat*-related gene, *vatB* and *satA* respectively and the gene *vgbB* presents 69.5 % nucleotides identity with the gene *vgb*.

VatC acetyltransferase exhibits significant similarity with acetyltransferases having the same enzymatic activity and encoded by the genes *vatC*, *vatB*, and *sat* (respectively 69.8, 58.2 and 66.0 % amino acids identity). These proteins belong to a family of xenobiotic acetyltransferases modifying various substrates including streptogramin A and related antibiotics. *VgbB* lactonase exhibits as well significant similarity with *Vgb* inactivating streptogramin B and related (67.0 % amino acids identity).

The two other genes carried by pIP1714 are *pre* and *repB*, encoding proteins involved in mobilization and replication, respectively. These two genes are homologous to those carried by the staphylococcal plasmid, pUB110 (McKenzie et al., 1986, Plasmid 15:93-103). Moreover, as reported in Figure 5, the

intergenic sequences of pIP1714 delimited by *vatC* and *repB* also exhibited significant similarities with pUB110.

Example 6: *Plasmid DNA isolation from PIIA^R staphylococci*

The staphylococci were grown after overnight incubation at 37°C in 200 ml BHI containing 10 µg/ml of PIIA. After 15 min centrifugation at 8000 rpm, the pellet was resuspended in 25 ml TES (Tris 50 mM, EDTA 1 mM, saccharose 7%). After adding 150 µg of lysostaphin, the mixture was incubated 30 min at 37°C. Then, 2ml of SDS 20% and 6 ml of EDTA 0.25 M were added and the suspension was incubated 15 min at 37°C. 8 ml of NaCl 5M were added and the mixture was kept 90 min at +4°C. After 30 min centrifugation at 8000 rpm, the supernatant was incubated 15 min at 37°C with 5 µg of Rnase (Boehringer). 10 µg of Proteinase K were added and the suspension was incubated 15 min at 65°C. DNA was precipitated using isopropanol (0.6 V for 1 V of DNA solution). After 30 min centrifugation at 8000 g, the pellet was washed with 10 ml ethanol 70%. The washed DNA was dried at 56°C, dissolved in 10 ml water and purified by dye-buoyant density centrifugation (ethidium bromide - cesium chloride). The extrachromosomal band was collected. After removing ethidium bromide, the solution of plasmid DNA was dialyzed using TE buffer (Tris, 10 mM, EDTA 1 mM, pH 7).

Example 7: *Plasmid DNA isolation from E. coli*

Cf. QIAfilter plasmid maxi protocol for large-scale preparations and QIAprep Spin plasmid kit protocol for mini-preparations.

Quiagen GmbH and Quiagen Inc. (Hilden, Germany)

- Plasmid maxi kit

Ref: 12262

- Miniprep kit

Ref: 27104

Example 8: *Transformation by electroporation of the S. aureus recipient strain, RN4220*

1 - Preparation of cells

200ml of BHI was inoculated with 20ml of an overnight culture of RN4220 (Kreisswirth et al., Nature 1983, 306:709-712) and incubated at 37°C with shaking. When the OD reached 0.4 at 600 nm, the suspension was kept in ice. The pellet was washed three times with 20 ml of cold Hepes buffer (saccharose 9.31 % - Hepes 0.19 % - pH. 7.4). The pellet was resuspended in 2.5 ml of Hepes buffer containing 10% glycerol. Aliquots of 100 µl cell suspension ($3 \cdot 10^{10}$ /ml) were stored at -80°C.

2 - Electroporation

After thawing at room temperature, the 100 µl aliquot of cells was kept in ice. After adding 10 µl of a solution

containing 1 μ g of plasmid DNA, the mixture was transferred to a cold 0.2 cm electroporation cuvette. The Gene Pulser (BioRad) was set at 25 μ F and 2.5 KV and the Pulse Controller to 100 Ω . This produced a pulse with a constant time of 2.3 to 2.5 m sec. The cuvette was removed from the chamber and 1 ml of SOC (2% bactotryptone, 0.5% bacto yeast extract, 10mM NaCl, 2.5mMKCl, 10mM MgCl₂, 10mM MgSO₄, 20 mM glucose) was added. The cell suspension was transferred in a propylene tube and incubated with shaking at 37°C for 1 hr. The suspension was then plated on selective medium, which consisted of BHIA containing 10 μ g/ml erythromycin or 10 μ g/ml of PIIA. The plates were incubated 48 h at 37°C and the transformants isolated on selective medium. The further studies were carried out on a single isolated colony.

Example 9: Polymerase chain reaction

DNA was amplified by PCR in a Crocodile 11 thermal cycler (Appligène) with approximately 10ng of cellular DNA or 1ng of plasmid DNA. The reaction mixture contained 0.6 μ M of each oligonucleotide serving as primer, 200 μ M of each deoxynucleotide triphosphate, 2.5 U of Taq DNA Polymerase (Amersham, Int.), and 1 x buffer (Amersham, Int.). The final reaction volume was adjusted to 100 μ l with H₂O and the sample was then covered by 50 μ l of heavy white mineral oil (Sigma Chemical Co, St. Louis, Missouri).

PCR experiments were carried out at high or low stringency, depending on the primers used. At high stringency, the PCR was performed with a precycle of 3 min at 95°C and 2 min at 60°C, 30 cycles of 20 sec at 72°C, 20 sec at 95°C, 20 sec at 60°C followed by a cycle of 1 min at 72°C. At low stringency, the PCR was performed with a precycle of 5 min at 95°C, 35 cycles of 2 min at 40°C, 1 min 30 sec at 72°C, 30 sec at 95°C followed by a cycle of 4 min at 40°C and 12 min at 72°C. The oligonucleotides used at high stringency are indicated in the Table below.

	PRIMER
vgaB	Oligo I 5'- <u>AAGTCGACT</u> GTACAATATGAGTGGTGG-3' Sali
	Oligo II 5'- <u>CTGCAGAT</u> GCCTCAACAGCATCGATATCC-3' PstI
vatC	Oligo III 5'- AT <u>GAATTC</u> GCAAATCAGCAAGG -3' EcoRI
	Oligo IV 5'- TCGTCTC <u>GAGCTC</u> TAGGTCC -3' SacI
vgbB	Oligo V 5'- CAGCAGT <u>CTAGAT</u> CAGAGTGG -3' XbaI
	Oligo VI 5'- CATAC <u>GGATCC</u> ACCTTTTCC -3' BamHI

Example 10: Labelling of DNA probes

Plasmid DNA was labelled with [α -³²P]dCTP (110 Tbq mmol⁻¹) by the random printing technique using the Megaprime DNA labelling system (Amersham).

Example 11: Blotting and hybridization

Hybond-N+membranes (Amersham) were used for blotting. DNA was transferred from agarose gels to the membranes by the capillary blotting method of Southern Blotting. DNA was denatured and fixed to the membranes according to the protocol described in the handbook user of Hybond-N+ membranes.

Prehybridization and hybridization were done at 68°C in a mixture containing 5X SSPE (1X SSPE is 0.3 M NaCl, 30 mM tri-sodium citrate), 5X Denhardt's solution, 0.5% (w/v) SDS, and 100 μ g ml⁻¹ salmon sperm DNA. The membranes containing DNA

transferred from agarose gels were treated with 10 ng ml⁻¹ radiolabeled DNA probe. Washing was started with two successive immersions in 2X SSPE, 0.1% SDS, at room temperature for 10 min, followed by one immersion in 1X SSPE, 0.1% SDS, at 68°C for 15 min, and finally by one immersion in 0.1 X SSPE, 0.1% SDS, at 68°C for 15 min. The washed blots treated with the radiolabeled probe were exposed to Fuji RX film at -70°C.

Example 12: Nucleotides sequence determination

For *vatC* and *vgbB*, the sequencing reaction was performed by PCR amplification in a final volume of 20 µl using 500 ng of plasmid DNA, 5-10 pmoles of primer and 9.5 µl of DyeTerminators premix according to Applied Biosystems protocol. After heating to 94°C for 2 min, the reaction was cycled as the following: 25 cycles of 30s at 94°C, 30s at 55°C, and 4 min at 60°C (9600 thermal cycler Perkin Elmer). Removal of excess of DyeTerminators were performed using Quick Spin columns (Boehringer Mannheim). The samples were dried in a vacuum centrifuge and dissolved with 4µl of deionized formamide EDTA pH 8.0 (5/1). The samples were loaded onto an Applied Biosystems 373A sequencer and run for 12 h on a 4.5% denaturing acrylamide gel.

- Primers used for sequencing the following genes:

• *vatC*

5'-GAAATGGTTGGGAGAAGCATACC-3'	5'-CAGCAATCGCGCCCGTTTG-3'
5'-AATCGGCAGAATTACAAACG-3'	5'-CGTTCCCAATTTCGGTGTACC-3'

• *vgbB*

5'-GTTTCTATGCTGATCTGAATC-3'	5'-GTCGTTTGTAAATTCTGCCGATT-3'
5'-GGTCTAAATGGCGATATATGG-3'	5'-TTCGAATTCTTTTATCCTACC-3'

For *vgaB*, DNA was sequenced according to the instructions provided by the T7Sequencing™ kit from Pharmacia Biotech (Uppsala, Sweden), procedures C and D.

- Primers used for sequencing the following genes:

• *vgaB*

5'-GCTTGGCAAAAGCAACC-3'	5'-TGAATATAGGATAG-3'
5'-TTGGATCAGGGCC-3'	5'-CAATTAGAAGAACCAC-3'
5'-CAATTGTTTCAGCTAGG-3'	5'-GAATTCATTCTATGG-3'
5'-TACACCATTTGTTACC-3'	5'-CAAGGAATGATTAAGCC-3'
5'-GATTCAGATGTTCCC-3'	5'-TCATGGTCGCAATG-3'
5'-GTTGCTTTCGTAGAAGC-3'	5'-GTTATGTCATCCTC-3'
5'-GGTTCATCTACGAGC-3'	5'-GGATATCGATGCTG-3'
5'-GCCAACTCCATTC-3'	5'-CCTAGCTGAACAATTG-3'
5'-GAAGGTGCCTGATCC-3'	5'-ATACTAGAAATGC-3'

Example 13: DNA cloning

A standard protocol was followed for cloning into the vector pOX7, also named pOX300, the 2.4 kb *Hind*ffl-*Hae*III fragment of pIP1633 carrying *vgaB* (Fig. 1) and the plasmid pIP1714 carrying

vatC and vgbB (Fig. 4), linearized by cleavage with *Hind*III. The vector DNA (10-20 μ g) and the plasmids used in cloning experiments were cleaved with the appropriate restriction enzymes (30 Units) and purified by GeneClean Kit (Bio 101, La Jolla, Calif.). To avoid religation, the vector cleaved with a single enzyme was dephosphorylated by 30 min incubation at 37°C with 5 Units of alkaline phosphatase. Ligation was carried out in a total reaction volume of 10 μ l containing 0.1 μ g of the vector, 0.1 μ g of the plasmid, 0.5 mM ATP, 1 X T4 DNA ligase buffer and 0.1 Weiss Unit of T4 DNA ligase. After overnight incubation at 16°C, 1 to 2 μ l of the ligation mixture are used for transforming competent *E. coli* and the transformants were selected on solid media containing 100 μ g/ml of ampicillin.

Example 14: Susceptibility to antimicrobial agents

Susceptibility to antimicrobial agents was determined with a disk diffusion assay and commercially available disks (Diagnostic Pasteur). Additional disks prepared in our laboratory contained streptogramin A (20 μ g) or streptogramin B (40 μ g).

- NCCLS: Performance standards for antimicrobial disk susceptibility test, 1984, Approved standard M2-A3, 4:369-402.
- ECCLS: Standard for antimicrobial susceptibility testing by diffusion methods, 1985, ECCLS Document, 5:4-14.

Minimal inhibitory concentrations (MICs) of antibiotics were determined by serial twofold dilutions of antibiotics in MHA (Ericson H.M. and S.C. Sherris, ActaPathol. Microbiol. Scand., 1971, Suppl. 217:Section B).

Despite the relatively low frequency of detection of SgA^R staphylococci (1-10%) (Loncle et al., 1993; Allignet et al., 1996), four genes encoding resistance to streptogramin A have been detected and other resistance gene(s) are suspected to be carried by staphylococci. Surprisingly, the present and previous studies (Allignet et al., 1996) indicate that staphylococcal plasmids carrying two genes encoding streptogramin A resistance by two distinct mechanisms (inactivation by acetyltransferases and increased efflux) are widespread among staphylococci (32 of the 48 plasmids investigated).

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WHAT IS CLAIMED IS:

1. A purified polynucleotide selected from the group consisting of:

- a) SEQ ID NO: 1 or a fragment derived from SEQ ID NO: 1 containing 15 to 40 nucleotides;
- b) SEQ ID NO: 2 or a fragment derived from SEQ ID NO: 2 containing 15 to 40 nucleotides;
- c) SEQ ID NO: 3 or a fragment derived from SEQ ID NO: 3 containing 15 to 40 nucleotides;
- d) SEQ ID NO: 11;
- e) SEQ ID NO: 12;
- f) SEQ ID NO: 13; and
- g) SEQ ID NO: 14.

2. The purified polynucleotide according to claim 1 selected from the group consisting of:

- a) SEQ ID NO: 1 or a fragment derived from SEQ ID NO: 1 containing 15 to 40 nucleotides;
- b) SEQ ID NO: 2 or a fragment derived from SEQ ID NO: 2 containing 15 to 40 nucleotides;
- c) SEQ ID NO: 11;
- d) SEQ ID NO: 12;
- e) SEQ ID NO: 13; and
- f) SEQ ID NO: 14.

3. A purified peptide encoded by a polynucleotide as claimed in claim 1.

4. A purified peptide selected from the group consisting of:

- a) SEQ ID NO: 4 or a fragment derived from SEQ ID NO: 4 containing at least 10 amino acids;
- b) SEQ ID NO: 5 or a fragment derived from SEQ ID NO: 5 containing at least 10 amino acids;
- c) SEQ ID NO: 6 or a fragment derived from SEQ ID NO: 6 containing at least 10 amino acids;
- d) SEQ ID NO: 7;
- e) SEQ ID NO: 8;
- f) SEQ ID NO: 9; and
- g) SEQ ID NO: 10.

5. A composition comprising at least one polypeptide encoded by a nucleotide sequence selected from the group consisting of *vgaB*, *vgbB*, and *vatC*.

6. A composition comprising at least one nucleotide sequence according to claim 1 that encodes resistance to streptogramins or induces streptogramin resistance in Gram-positive bacteria.

7. The composition according to claim 6, wherein said composition comprises at least a nucleotide sequence encoding a molecule containing ATP binding motifs conferring resistance to a

streptogramin and at least one nucleotide sequence selected from the group consisting of:

- a) a nucleotide sequence encoding an acetyltransferase that confers resistance to streptogramin A; and
- b) a nucleotide sequence encoding a lactonase that confers resistance to streptogramin B.

8. The composition according to claim 6, wherein said composition comprises at least one nucleotide sequence encoding an acetyltransferase that confers resistance to a streptogramin and at least one nucleotide sequence selected from the group consisting of:

- a) a nucleotide sequence encoding a molecule containing ATP binding motifs that confers resistance to streptogramin A; and
- b) a nucleotide sequence encoding a lactonase that confers resistance to streptogramin B.

9. The composition according to claim 6, wherein said composition comprises at least one nucleotide sequence encoding a lactonase conferring resistance to a streptogramin and at least one nucleotide sequence selected from the group consisting of:

- a) a nucleotide sequence encoding a material containing ATP binding motifs that confers resistance to streptogramin A; and

b) a nucleotide sequence encoding an acetyltransferase that confers resistance to streptogramin A.

10. A composition comprising a polynucleotide sequence that encodes a molecule containing ATP binding motifs, which confer resistance to a streptogramin in *Staphylococcus* and wherein the polynucleotide sequence is selected from the group consisting of:

- a) SEQ ID NO: 1;
- b) a polynucleotide sequence having at least 70% of identity with SEQ ID NO: 1;
- c) a polynucleotide sequence hybridizing with said SEQ ID NO: 1 under stringent conditions or to a fragment containing between 20 and 30 nucleotides of SEQ ID NO: 11 or SEQ ID NO: 12;
- d) a polynucleotide sequence that encodes a polypeptide having at least 60% homology with SEQ ID NO: 4; and
- e) a polynucleotide sequence having at least 80% homology with SEQ ID NO: 11 or SEQ ID NO: 12.

11. A composition of claim 10, wherein the polynucleotide sequence encoding a molecule containing ATP binding motifs confers resistance to a streptogramin in *Staphylococcus aureus*.

12. A composition comprising a polynucleotide sequence that encodes an acetyltransferase, which confers resistance to a streptogramin in *Staphylococcus* and wherein the polynucleotide sequence is selected from the group of:

- a) SEQ ID NO: 2;
- b) a polynucleotide sequence having at least 70% of identity with SEQ. ID NO: 2;
- c) a polynucleotide sequence hybridizing with said SEQ ID NO: 2 under stringent conditions, or to a fragment of SEQ ID NO: 2 containing between 20 and 30 nucleotides, or to SEQ ID NO: 13 or SEQ ID NO: 14;
- d) a polynucleotide sequence that encodes a polypeptide sequence having at least 60% homology with SEQ ID NO: 5; and
- e) a polynucleotide sequence having 80% homology with SEQ ID NO: 13 or SEQ ID NO: 14.

13. A composition of claim 12, wherein the polynucleotide sequence encoding an acetyltransferase confers resistance to a streptogramin in *Staphylococcus cohnii*.

14. A composition comprising a polynucleotide sequence that encodes a lactonase, which confers resistance to a streptogramin in *Staphylococcus* and wherein the polynucleotide sequence is selected from the group consisting of:

- a) SEQ ID NO: 3;
- b) a polynucleotide sequence having at least 70% of identity with SEQ ID NO: 3;

- c) a polynucleotide sequence hybridizing with said SEQ ID NO: 3 under stringent conditions, or to a fragment of said SEQ ID NO: 3 containing 20 to 40 nucleotides; and
- d) a polynucleotide sequence that encodes a polypeptide having at least 60% homology with SEQ ID NO: 6.

15. A composition of claim 14, wherein the polynucleotide sequence encoding a lactonase confers resistance to a streptogramin in *Staphylococcus cohnii*.

16. A composition of polynucleotide sequences according to claim 7 or 8, wherein said composition comprises at least one nucleotide sequence encoding a molecule containing ATP binding motifs conferring resistance to a streptogramin, and at least one nucleotide sequence encoding an acetyltransferase conferring resistance to a streptogramin.

17. A composition of polynucleotide sequences according to claim 16, wherein said composition comprises at least one *vgaB* nucleotide sequence encoding a molecule containing ATP binding motifs conferring resistance to a streptogramin, and at least one *vatC* nucleotide sequence encoding an acetyltransferase conferring resistance to a streptogramin.

18. A purified polynucleotide that hybridizes specifically under stringent conditions with a polynucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2,

SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, and
SEQ ID NO: 14.

19. A polynucleotide fragment comprising at least 10 nucleotides that hybridizes under stringent conditions with a sequence according to claim 1 or the polynucleotide complementary fragment thereof.

20. A polynucleotide fragment of claim 19, wherein said fragment is selected from the group consisting of:

- Oligo I: 5'-AAGTCGACTGACAATATGAGTGGTGG-3';
- Oligo II: 5'-CTGCAGATGCCTCAACAGCATCGATATCC-3';
- Oligo III: 5'-ATGAATTCGCAAATCAGCAAGG-3';
- Oligo IV: 5'-TCGTCTCGAGCTCTAGGTCC-3';
- Oligo V: 5'-CAGCAGTCTAGATCAGAGTGG-3'; and
- Oligo VI: 5'-CATACGGATCCACCTTTTCC-3'.

21. The polynucleotide fragment of claim 19, wherein said fragment is selected from the group consisting of:

- Oligo I: 5'-AAGTCGACTGACAATATGAGTGGTGG-3';
- Oligo II: 5'-CTGCAGATGCCTCAACAGCATCGATATCC-3';
- Oligo III: 5'-ATGAATTCGCAAATCAGCAAGG-3'; and
- Oligo IV: 5'-TCGTCTCGAGCTCTAGGTCC-3'.

22. A recombinant DNA molecule comprising at least one nucleotide sequence according to claim 1 under the control of regulatory elements that regulate the expression of resistance to antibiotics of the streptogramin family in a host.

23. A recombinant vector comprising a plasmid pIP1633 deposited with the C.N.C.M. under Accession No. I-1768 or a plasmid pIP1714 deposited with the C.N.C.M. under Accession No. I-1877.

24. A recombinant cell host comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, and SEQ ID NO: 14, or the recombinant vector of claim 23.

25. A purified polypeptide or a peptide fragment having at least 10 amino acids, which is recognized by antibodies directed against a peptide encoded by a polynucleotide sequence conferring resistance to a streptogramin corresponding to a polynucleotide sequence according to claim 1.

26. A polynucleotide comprising a full length coding sequence of a *Staphylococcus* streptogramin resistant gene containing a polynucleotide sequence according to claim 1.

27. A monoclonal or polyclonal antibody directed against a polypeptide or a peptide fragment encoded by a polynucleotide sequence according to claim 1.

28. A method of detecting a bacterium in a biological sample that harbors a polynucleotide sequence according to claim 1, said method comprising the steps of:

- a) contacting bacterial DNA of the biological sample with a primer or a probe according to claim 19 or

20, which hybridizes with a nucleotide sequence encoding resistance to streptogramins;

- b) amplifying the nucleotide sequence using said primer or said probe; and
- c) detecting a hybridized complex formed between said primer or probe and the DNA.

29. A kit for detecting a bacterium that is resistant to a streptogramin and harbors a polynucleotide sequence according to claim 1, said kit comprising:

- a) a polynucleotide probe according to claim 19 or 20; and
- b) reagents to perform a nucleic acid hybridization reaction.

30. A kit for detecting a bacterium that is resistant to a streptogramin and harbors a polynucleotide sequence according to claim 2, said kit comprising:

- a) a polynucleotide probe according to claim 19 or 20; and
- b) reagents to perform a nucleic acid hybridization reaction.

31. A method of screening an active antibiotic for treating a Gram-positive bacterial infection, comprising the steps of:

- a) contacting the antibiotic with Gram-positive bacteria that are resistant to a streptogramin and contain a polynucleotide sequence according to claim 1; and
- b) determining the activity of the antibiotic on the bacteria.

32. A method of screening for active synthetic molecules capable of penetrating into a bacteria of the staphylococci family, wherein an inhibiting activity of the molecules is tested on at least a polypeptide encoded by a polynucleotide sequence according to claim 1, the method comprising the steps of:

- a) contacting a sample of said active molecules with the bacteria;
- b) testing the capacity of the active molecules to penetrate into the bacteria and the capacity of inhibiting a bacterial culture at various concentration of the molecules; and
- c) choosing the active molecule that provides an inhibitory effect of at least 80% on the bacterial culture compared to an untreated culture.

33. An *in vitro* method of screening for active molecules capable of inhibiting a polypeptide encoded by a polynucleotide sequence according to claim 1, said method comprising the steps of:

- a) contacting the active molecules with said polypeptide;
- b) testing the capacity of the active molecules, at various concentrations, to inhibit the activity of the polypeptide; and
- c) choosing the active molecule that provides an inhibitory effect of at least 80 % on the activity of the said polypeptide.

34. A composition of a polynucleotide sequence encoding resistance to streptogramins, or inducing resistance in Gram-positive bacteria, wherein said composition comprises a nucleotide sequence corresponding to a resistance phenotype of a plasmid selected from the group consisting of:

- a) plasmid pIP1633 deposited with the C.N.C.M. under Accession No. I-1768;
- b) plasmid pIP1680 deposited with the C.N.C.M. under Accession No. I-1767; and
- c) plasmid pIP1714 deposited with the C.N.C.M. under Accession No. I-1877.

35. A method of detecting a bacterium in a biological sample that harbors a polynucleotide sequence according to claim 2, said method comprising the steps of:

- a) contacting said sample with an antibody according to claim 27 that recognizes a polypeptide encoded by said polynucleotide sequences; and
- b) detecting a complex formed between the antibody and the polypeptide.

36. A diagnostic kit for *in vitro* detection of a bacterium harboring the polynucleotide sequences according to claim 2, said kit comprising:

- a) a predetermined quantity of monoclonal or polyclonal antibodies according to claim 27;
- b) reagents to perform an immunological reaction between the antibodies and a polypeptide encoded by said polynucleotide sequences; and
- c) reagents for detecting a complex formed between the antibodies and the polypeptide encoded by said polynucleotide sequences.

ABSTRACT

The present invention pertains to polynucleotides derived from *staphylococcal* genes encoding resistance to streptogramin A or to streptogramin B and chemically related compounds. This invention also relates to the use of the polynucleotides as oligonucleotide primers or probes for detecting *Staphylococcal* strains that are resistant to streptogramin A or to streptogramin B and related compounds in a biological sample. In another embodiment, the present invention is directed to the full length coding sequences of the *staphylococcal* genes encoding for resistance to streptogramin A or to streptogramin B from *Staphylococcus* and to the polypeptides expressed by these full length coding sequences. Further, this invention relates to the use of the expressed polypeptides to produce specific monoclonal or polyclonal antibodies that serve as detection means in order to characterize any *staphylococcal* strain carrying genes encoding resistance to streptogramin A or to streptogramin B.

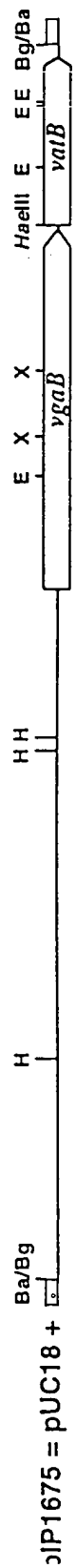
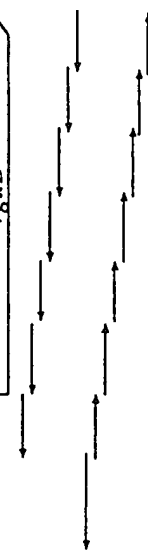


Figure 1A



Figure 1B



1000 bp

aaactttaacttaagtagcagaagattatggagtaataactaagaacaagtgtatgtactaaagaacaaagcctaaagatgatatattgacttattataaagaagcaat 120
HindIII
tgaatatganaagatcgaaatgaaaaactatcaaaaacttaacgataatttatttggaaacagtttagatanaaaatcaaacattattagatcagcaacaagaattaaagtcttaattgatcaaa 240
atagtatcanaaagttagaatacgaattagaagaaaaaaagaagaaaaaagaactaagtgggtatccatgtatccagagaaaaaaataattatataatbaaaatgagatacaa 360
caaatgaattggttcttcaatagggaatttggtaaaacccatgtacatatcaacttttaatttagtataatttaaaatacaaacgaaggaatcgaaggaatataaataaataatgatcaaa 480
taatccgctcactaaaaaagaataatatttggaagattttacctaataattattgtctattattatgtaggtttgtttattgttataatttccatttatataaaactatcc 600
tctattttaaaaaaggaggattttttttatgcttttggttatttttgcattatataaaactatccctctatttttaaaaaaaggaggatttttttatgctttaaaaatcgacatgaag 720
N V K K Y Y A D K L I L N I K E L K I Y S G D K I G I V Q K N G V G K T T L L K
aatgtaaaaaaatattatgcagatanaatttaatttcaataataaaagaactaaagatttatttagtgggataaaaataggtattgttagtaagaatggagttggcaaacacacacttttcaaaa 840
I I K G L I E I D B G N I I I S E K T T I K Y I S Q L E B P H S K I I D G K Y A
ataataaaaggactaatagagattgcgaaggaataataattatgaagtgaaaaaaactatttaataataatctctcaattagaagaaacccacatagtaagataaattgatggaaaaatagctt 960
S I F Q V R N K W N D N M S G G E K T R F K L A E G F Q D Q C S L H L V D E P T
tcaatatttcaagttgaaaaaataagtggaatgacaatagtggtgggtgaaaaaaactagatttaaaactagcagagggatttcaagatcaaatgttctttaaagtcgttagatgaacctaca 1080
S N L D I E G I E L I T N T P K E Y R D T F L V S H D R I F L D Q V C T K I P
agtaatttagatcgaaggaataggttgataacaaataactttttaaagtagtcgtagacttttttggtagtactcatgatagaatttttttagatcaagtttctgtacaaaaattttt 1200
B I E N G Y I R E F I G N Y T N Y I E Q K E N L L R K Q Q E Y E K Y N S K R K
gaattgaaaatggatatattaggaattcatcggttaattatacaaaactatatagagcaaaaagaatgcttctcgaagaaacgaagaatacgaagaaataattctaaagaagaag 1320
EcoRI
Q L E Q A I K L K B N K A Q Q M I K P P S K T M G T S E S R I N K M Q H A T K Q
caattggagcagctataaagctaaagagaataaggcgaaggaatgattaaagcccttcaaaaaaatgggaacatctgaattctagaataatggaaaantggcaantgctactaaacaa 1440
XbaI
K K M H R N T K S L E T R I D K L N H V E K I K E L P S I K M D L P N R E Q P H
aaaaagatgcatagaatacgaatacgttggaaacacgaatagataaaatnaantcatgtagaaaaataaaagagcttcttctattaaantggattacctaataagagagcaatttcat 1560
G R N V I S L K N L S I K P N N Q F L W R D A S P V I K G G E K V A I I Q N N Q
ggtcgcaatgaattagtttaaaaaacttatctataaaatttaataatcaatttcttggagagatgcttcatcttaaaagtggaagaaaggttgctataattgggtaacaatggt 1680
V Q K T T L L K L I L E K V E S V I I S P S V K I G Y V S Q N L D V L Q S H K S
gtaggaaaacaacacattgttgagctgattctagaaaaagtagaatcagtaataatcaccatcagttaaaaattggatcgtcagcaaaattagatgttctacaaatctcataaatct 1800
XbaI
I L E N V M S T S I Q D E T I A R I V L A R L H P Y R N D V H K E I N V L S G G
atcttagaaaatgttatgtctaccctcattcaagatgaacaatagcaagaattgttcttagcaagattacatttttctatcgcaatgtgttctataaagaataaaatgttttgagtcggga 1920
B Q I K V A F A K L P V S D C N T L I L D B P T N Y L D I D A V E A L B E L L I
gaacaaataaaggttcttggccaagctatttggtagcgatttgaatacatatttcttgatgaaccaaaactatttggatcgtatgctgttgaggcattagaagaattgttaatt 2040
T Y E G V V L F A S H D K K P I Q N L A E Q L L I I E N N K V K K P E Q T Y I E
acctatgaaggttgtgttatttgcctcccatgataaaaaatttatcaaaaacttagtgaaacttgttaataatagaaaaataaagaatgaaaaaatcgaaggaacatatatagaa 2160
Y L K I K D K P K L N T N B K E L K E K M I L E M Q I S S L L S K I S M E E N
tatttaaaataaagntaaacaaaatttaatacaaaatgnaaaagaactcnaagaaaaaaagatgatactaganaatgcaaaatttccattatttaagtaaaaaatctcaatgggaagaaat 2280
E E K N K E L D E K Y K L K L K B L K S L N K N I
gaagaaaaaacaaagaattagatgaaaaagtacaaaattgaaaataaagaattgaaaagcctaanaataaaaaatatttcaaaaataattatataatagagggttcaaaaatgaaatctggcc 2400
P D P N
ctgctcccaat
M K Y G
StartvalB HaeIII
EndvgaB
2411

Figure 2

		WA	
vgaB	-MLKIDMKNVKKYYADKLILNIKELKIYSGDKIGIVGKNGVGKTTLLKIIKGLIEIDEGN		59
vga	MKIMLEGLNIKHVQDRLLLNNRLKIYQNDRIGLIGKNGSGKTTLLHILYKKIVPEEG-		59
	. . . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * .		
		loop 3	
vgaB	IIISEKTTIKYISQLEEPHSKIIDGKYASIFQVENKWNDNMSGGEKTRFKLAEGFQDQCS		119
vga	-IVKQFSHCELIPQLKLIES-----TKSGGEVTRNYIRQALDKNPE		99
	* . . . * * * * . * . * . * . * . * . * . * . * . * . * .		
	WB		
vgaB	LMLVDEPTSNLDIEGIELITNTFKEYRDTFLVVSHDRIFLDQVCTKIFEIENGYIREFIG		179
vga	LLLADEPTTNLDNNYIEKLEQDLKNWHGAFIIVSHDRAFLDNLCCTTIWEIDEGRITEYKG		159
	* . * * . * . * . * . * . * . * . * . * . * . * . * . * . * .		
vgaB	NYTNYIEQKEMLLRKQQEYEEKYNSKRKQLEQAIIKLKENKAQGMKPPSKTMGTSES--R		237
vga	NYSNYVEQKELERHREELEYEKEYEKKRLEKAINIKEQKAQRATKKP-KNLSLSEGGIK		218
	** . * . * . * . * . * . * . * . * . * . * . * . * . * . * .		
vgaB	IWKMQHATKQKKMHRNTKSLETRIDKLNHVEKIKELPSIKMDLPNREQFHGRNVISLKNL		297
vga	GAKPYFAGKQKKLRKTVKSLETRLEKLESVEKRNELPPLKMDLVNLESVKNRITIIRGEDV		278
	* * * * . * . * . * . * . * . * . * . * . * . * . * . * .		
		WA	
vgaB	SIKFNNQFLWRDASFVIKGGKVAIIIGNNGVGKTTLLKLILEKVESVIIISPSVKIGYVSQ		357
vga	SGTIEGRVLWKAKSFSIRGGDKMAIIGSNGTGKTTFIKKIVHGNPGISLSPSVKIGYFSQ		338
	* . . * . * . * . * . * . * . * . * . * . * . * . * . * .		
		loop 3'	
vgaB	NLDVLQSHKSILENVMSTSIQDETIARIVLARLHFYRNDVHKEINVLSGGEQIKVAFKL		417
vga	KIDTLELDKSILENVQSSSQONETLIRTIARMHFFRDDVYKPISVLSGGERVKVALTKV		398
	* * . * . * . * . * . * . * . * . * . * . * . * . * . * .		
	WB		
vgaB	FVSDCNTLILDEPTNYLDIDAVEALEELLITYEGVVLFAASHDKKFIQNLAEQLLIIENNK		477
vga	FLSEVNTLVLDEPTNFLDMEAIEAFESLLKEYNGSIIFVSHDRKFIEKVATRIMTIDNKE		458
	* . * . * . * . * . * . * . * . * . * . * . * . * . * . * .		
vgaB	VKKFEGTYIEYLKIKDKPKLNTNEKELKEKKMILEMQISSLLSKISMEENEKKNKELDEK		537
vga	IKIFDGTY-EQFKQAEKPTRNIKE----DKLLLETKITEVLSRLSIEPSEE----LEQE		509
	* * . * . * . * . * . * . * . * . * . * . * . * . * . * .		
vgaB	YKLKLEKLSLNKNI		552
vga	FQNLINELKRNLDK--		522
	* * *		

Restriction map of pIP1714

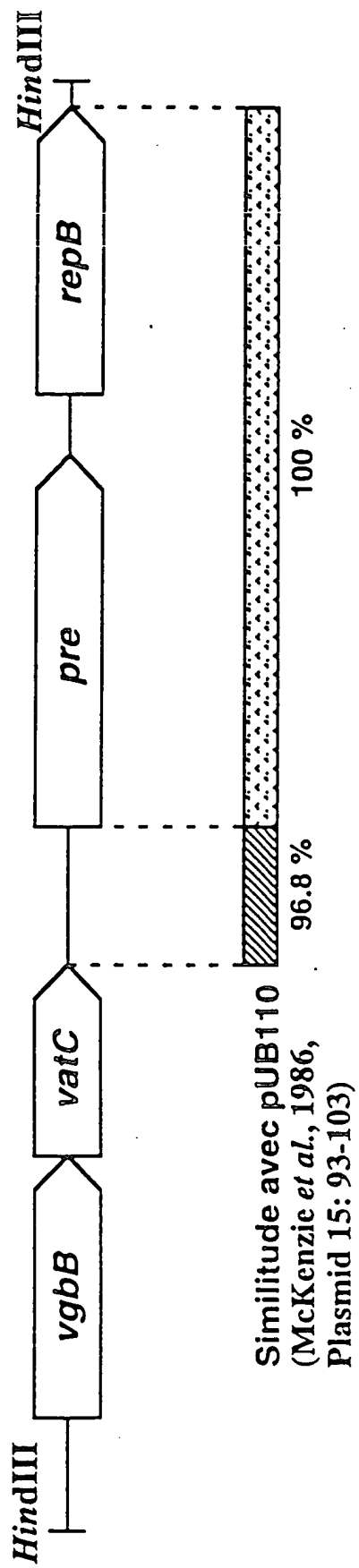


Figure 4

PCR Primers

vatC

Oligo III 5'- atgaattcgcaaatacagcaagg-3'
 EcoRI

Oligo IV 5'- tcgtctcgagctctaggtcc-3'
 SacI

Figure 6A

vgbB

Oligo V 5'- cagcagctctagatcagagtgg-3'
 XbaI

Oligo VI 5'- catacggatccacctttcc-3'
 BamHI

Figure 6B

vga B

Oligo I 5' -AAGTCGACTGACAATATGAGTGGTGG- 3'

Oligo II 5' -CTGCAGATGCCTCAACAGCATCGATATCC- 3'

Figure 6C

SEQ ID NO: 1

Seq vgaB

ATGCTTAAATCGACATGAAGAATGTAAAAAATATTATGCAGATAAATTAATTTTAAATATAAA
AAAGATTTATAGTGGGGATAAAATAGGTATTGTAGGTAAGAATGGAGTTGGCAAAACAACACTTT
TAATAAAGGACTAATAGAGATTGACGAAGGAAATATAATTATAAGTGAAAAACAACCTATTAAA
TCTCAATTAGAAGAACCACATAGTAAGATAATTGATGGAAAATATGCTTCAATATTTCAAGTTGA
GTGGAATGACAATATGAGTGGTGGTGAAAAAAGTAGATTTAACTAGCAGAGGGATTTCAGATC
CTTTAATGCTCGTAGATGAACCTACAAGTAATTTAGATATCGAAGGAATAGAGTTGATAACAAAT
AAAGAGTACCGTGATACTTTTTTGGTAGTATCTCATGATAGAATTTTTTTAGATCAAGTTTGTAC
TTTTGAAATTGAAAATGGATATATTAGAGAATTCATCGGTAATTATACAACTATATAGAGCAAA
TGCTTCTACGAAAGCAACAAGAAGATACGAAAGTATAATTCTAAAAGAAAGCAATTGGAGCAA
AAGCTAAAAGAGAATAAGGCGCAAGGAATGATTAAGCCCCCTTCAAAAACAATGGGAACATCTGA
AATATGGAAATGCAACATGCTACTAAACAAAAAAGATGCATAGAAATACGAAATCGTTGGAAA
TAGATAAATTAATCATGTAGAAAAAATAAAAGAGCTTCCTTCTATTAAATGGATTTACCTAAT
CAATTTTATGGTTCGCAATGTAATTAGTTTAAAAAAGCTTATCTATAAAATTTAATAATCAATTTCT
AGATGCTTCATTTGTCAATTAAGGTGGAGAAAAGGTTGCTATAATTGGTAAACAATGGTGTAGGAA
CATTGTTGAAGCTGATTCTAGAAAAGTAGAATCAGTAATAATATCACCATCAGTTAAAATTGGA
AGTCAAACTTAGATGTTCTACAATCTCATAAATCTATCTTAGAAAATGTTATGTCTACCTCCAT
TGAAACAATAGCAAGAATTGTTCTAGCAAGATTACATTTTTATCGCAATGATGTTCTATAAGAAA
TTTTGAGTGGTGGAGAACAAATAAAGGTTGCTTTTGCCAAGCTATTTGTTAGCGATTGTAATACA
CTTGATGAACCAACAACTATTTGGATATCGATGCTGTTGAGGCATTAGAAGAATTGTTAATTAC
AGGTGTTGTGTTATTTGCTTCCCATGATAAAAAATTTATACAAAACCTAGCTGAACAATTGTTAA
AAAAATAATAAGTGAAAAAATTCGAAGGAACATATATAGAATATTTAAAAATTAAAGATAAACCA
AATACAAATGAAAAAGAACTCAAAGAAAAAAGATGATACTAGAAATGCAATTTTCATCATTATT
AATCTCAATGGAAGAAAATGAAGAAAAAACAAGAATTAGATGAAAAGTACAAATTGAAATTAA
TGAAAAGCCTAAATAAAAAATATT

SEQ ID NO: 3

Seq vgbB

ATGAATTTTTIATTTAGAGGAGTTTAACTTGTCTATTCCCGATTGAGGTCCATACGGTATAACTTC
AGACGGAAAGGTATGGTTCAACAACATAAGGCAACAAAATCAGCAGTCTAGATCAGAGTGGTA
AAGAATTCGAAGTTCCTACCCCTGATGCTAAAGTGATGTGTTAATTGTATCTTCACTTGGAGAC
TTTACAGAGAATGGTGCAATAAAATCGGAAAGCTCTCAAAAAAGGTGGCTTTACAGAATATCC
ACAGCCGGATTCTGGTCCTTACGGAATAACGGAAGGTCTAAATGGCGATATATGGTTTACCCAAT
GAGATCGTATAGGAAAGTTGACAGCTGATGGGACTATTTATGAATATGATTTGCCAAATAAGGGA
CCTGCTTTTATTACTTTAGGTTCCGATAACGCACTTTGGTTTACGGAGAACCAAAATAATTCTAT
GATTACAAATACAGGGAATTAGAAGAATATCTCTACCAACAAATGCAGCGGCTCCAGTGGGTA
GTGGTAACGATGGTGCACTCTGGTTTGTGCAAAATTATGGGCAACAAAATAGGTGGAATCACTACA
GAGATTAGCGAATATGATATTTCAACTCCAAACGCACGTCCACACGCTATAACCGCGGGGAAAA
AATATGGTTTACTGAATGGGGGGCAAATCAAATCGGCAGAATTACAAACGACAAAACAATTCAAG
AACTTCAAACAGAAAATGCGGAACCTCATGGTATTACCTTTGGAAAAGATGGATCCGTATGGTTT
AAATGTAAAATTGGGAAGCTGAATTTGAACGAA

Figure 7

SEQ ID NO: 2

Seq varC

ATGAAATGGCAAAATCAGCAAGGCCCAATCCAGAAGAAATATACCCCTATAGAAGGTAATAAAC
AATTTATTAAACCATCTATAACAAAGCCCAATATTTTAGTTGGGGAATATTATATTACGATAG
TGGTGAATCTTTTGAAAGCCAAGTTCTTTATCACTATGAATTGATTGGGGATAAACTAATATTA
TTTTGTTCTATTGGACCCGGAACGACATTTATAATGAATGGGGCTAATCATCGTATGGATGGTT
TTCCATTCAATCTTTTCGGAAATGGTTGGGAGAAGCATACCCCTACATTGGAAGACCTTCCTTA
TAACACGGAAATTGGGAACGATGTTTGGATTGGACGAGATGTGACAATTATGCCCGGTGTAAAA
AACGGGGCTATTATTGCAGCAAAATCGGTTGTGACAAAGAACGTTGATCCTTATTGAGTTGTTG
ATCCTTCACGATTAATTAAGATAAGGTTTTCCAAGGAAAAAATCGCAGCATTACTAAAAGTAAG
GGACCTAGAGATAGAGACGATAAATGAAAATATTGATTGCATCCTGAATGGTGATATAAAAAAG
AGAAGT

Figure 7 (cont.)

SEQ ID NO: 4

VgaB

MLKIDMKQVVKYYADKLIILNIKELKIYSGDKIGIVGKNGVGKTTLLKIIK
GLIEIDEGNIIISEKTTIKYISQLEEPHSKIIDGKYASIFQVENKWNNDNM
SGGEKTRFKLAEGFQDQCSLMLVDEPTSNLDIEGIELITNTFKEYRDTFL
VVSHDRIFLDQVCTKIFEIENGYIREFIGNYTNYIEQKEMLLRRQQEYE
KYNKRKQLEQAIKLKENKAQGMKPPSKTMTGTSESRIWQMQHATKQKQM
HRNTKSLETRIDKLNHVEKIKELPSIKMDLPNREQFHGRNVISLNLNLSIK
FNNQFLWRDASFVIKGGKVAIIIGNNGVGKTTLLKLILEKVESVIISPSV
KIGYVSQNLVDLQSHKSILENVMSTSIQDETIARIVLARLHFYRNDVHKE
INVLSGGEQIKVAPAKLPVSDCNTLILDEPTNYLDIDAVEALEELLITYE
GVVLFASHDKKFIQNLAEQLLIIENNKVKKFEGTYIEYLKIKDKPKLNTN
EKELKEKGMILEMQISSLLSKISMEENEEKNKELDEKYKLKLKELKSLNK
NI

SEQ ID NO: 6

VgbB

MNFYLEEFNLSIPDSGPYGITSSDGKVVFTQHKANKISSLDQSGRIKEF
EVFTPDAKVMCLIVSSLGDIWFTENGANKIGKLSKGGFTEYPLPQPSG
PGITEGLNGDIWFTQLNGDRIGKLTADGTIYEYDLPNKGSYPAPITLGS
NALWFTENQNNSIGRITNTGKLEEYPLPTNAAAPVGITSGNDGALWFVEI
MGNKIGRITTTGEISEYDIPTPNARPHAITAGKNSEIWFTWGANQIGRI
TNDKTIQEYQLQTENAEPEGITFGKDGSVWPAKCKIGKLNLE

SEQ ID NO: 5

VatC

MRWQNOQGPNPBBIYPIEGNKHVQFIKPSITKPNILVGEYSYDSKDGES
FESQVLYHYELIGDKLILGKFCSIGPGTTFIMNGANHRMDGSTFPFNLF
NGWEKHTPTLEDLPYKGNTEIGNDVWIGRDVTIMPGVKIGNGAIIAAKSV
VTKNVDPYSVVGGNPSRLIKIRFSKERIAALLKVRNWDLEIETINENIDC
ILNGDIKKVKRS

Figure 7 (cont.)

SEQ ID NO: 7	K S I L E N V	VgaB
SEQ ID NO: 11	1795 5'- aaa tct atc tta gaaaat gtt -3' g agc t	vgaB

SEQ ID NO: 8 N Y T N Y I E Q K E VgaB
 S V

SEQ ID NO: 12 1237
5'-aat tat aca aac tat ata gag caa aaa gaa-3' 1266
 gt gtt a vgaB

SEQ ID NO: 9 I M N G A N H R M

SEQ ID NO: 13

1187 'a c a a 1213
5'- ata atg aat ggg gct aat cat cgt atg -3' vatC
 t t a c g

EQ ID NO: 10 G N D V W I G VatC

EQ ID NO: 14 1310 a t g t 1330
5'- ggg aac gat gtt tgg att gga -3' **vatC**
 a t a a t